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Ultrastructural Analysis of Neurosecretory Cells in the Antennae of the Mosquito, *Culex Salinarius* (Diptera: Culicidae)

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Abstract

An antiserum raised against the peptide, culetachykinin II, immunocytochemically detected a group of neurosecretory cells in the first flagellar segment of the antennae of both males and females of the mosquito, *Culex salinarius*. This is the first insect species in which neurosecretory cells have been found in the antennae. The ultrastructure of these antennal neurosecretory cells (ANC) is described, as well as their relationship to other neurons in the antennae and antennal lobe of the mosquito. These tachykinin-reactive cells contain relatively small (140–220 nm) elementary neurosecretory granules. Not only do the ANC have axons that terminate on specific glomeruli of the deutocerebrum, but these neurons also have collaterals that form neurohemal terminals in the receptor lymph channels of the dendrites of the sensory neurons. Thus, the ANC not only influence higher centers of the brain that interpret signals from the antennal sensillae, but also modulate the response of the sensory receptors. To our knowledge, this is the first report of neurosecretory cells directly affecting the signal reception of sensory neurons.

Index Entries: Sensory neurons; neurohormones; peptide; tachykinin; chemosensilla.

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Introduction

The sense of smell is one of the most primitive senses that has been conserved throughout phyla, linking the brain with the external environment. In insects, it is required for finding food, mates, and oviposition sites. Recently, an olfactory sensory map has been devised for mammals in which odor molecules that interact with receptors activate sites in the olfactory bulb of the brain (Vassar et al., 1994). The olfactory bulb acts as a relay site through internuncial nerves that are connected to other portions of the brain where the olfactory signals are analyzed and trigger motor neuron responses. An analogous type of configuration has also been described for insects by several other scientists (*see* reviews by McIver, 1982; Keil and Steinbrecht, 1994; Zacharuk, 1985; Hilebrand and Shepard, 1997), and investigators of olfactory nervous systems of insects have reported a uniform system throughout the classes of insects studied thus far (Todd and Baker, 1997). The basic unit of this system consists of a series of cuticular sensillae containing one or more sensory dendrites that extend from the surface of the antennae. Chemical signals received from the environment by the dendrites are transmitted to the axons of these sensory receptor cells to specific sites in the antennal lobes of the insect (McIver, 1982). In the antennal lobes, these signals are relayed to interneurons, where they are carried to higher centers of the brain and integrated with sensory information from other sources (Bowen, 1991; Todd and Baker, 1997).

During a recent study in which we localized the sites of synthesis of a tachykinin-related peptide, isolated from the mosquito, *Culex salinarius* (Meola et al., 1998), we found that the antennal sensory system of this species is more complex than that previously reported in insects. In addition to culetachykinin-reactive cells in the brain and intestine, the first flagellar segment of the antennae of both male and female mosquitoes of this species contained neurons immunoreactive to this peptide. This was an important discovery, because our laboratory has been involved in developing mimetics of insect peptides as potential control agents for pest species of insects (Abernathy et al., 1996). *Culex salinarius* occurs throughout the United States and is most abundant in the Atlantic and Gulf Coast regions and has been implicated as a possible vector of eastern equine encephalitis and St. Louis encephalitis viruses as well as the causative agent of dog heartworm (Tveten and Meola, 1988). Thus an ultrastructural study was undertaken to determine the structure of these neurons and their relationship to the sensory receptors of the antennae and to the central nervous system (CNS).

Materials and Methods

Experimental Animals

Adult male and female *C. salinarius* used in the immunocytochemical study were obtained from the same wild populations collected in the Anahuac National Wildlife Refuge in Chambers County, TX, as those used in the isolation of the peptide (Meola et al., 1998). Wild populations were used because laboratory colonies of this species could not economically supply the number of mosquitoes required for peptide extraction.

Adult male and female *C. salinarius* used in the ultrastructural study were obtained from colonies reared at the Department of Entomology, Texas A&M University. The mosquitoes were reared in an environmental chamber at 20°C and 80% relative humidity on a 9 : 13 L : D photoperiod regime. Larvae were fed daily with a slurry containing finely ground TetraMin® fish food (TetraWerks, Melle, Germany). Dry Bermuda grass, *Cynodon dactylon* L., was added to the larval rearing trays to provide supplement food and protective shelter. The adults were maintained on 10% sugar water as a carbohydrate source and live chickens were hosts for the blood meals required by the females for egg development. The adults used in the ultrastructural study were 3 d post-emergence. Mosquitoes were sacrificed for optical and electron microscopy procedures after anesthesia in an atmosphere of carbon dioxide.

Experimental Procedure

Immunocytochemistry

Procedures used were described by Meola et al. (1998). Briefly, whole bodies were fixed in aqueous Bouins (Humason, 1962), dehydrated in ethanol, cleared in xylene, and embedded in low temperature (50–54°C) paraplast (Oxford Labware, St. Louis, MO). Specimens were sectioned at 5 μm and processed for immunocytochemistry as described by Meola et al. (1991). The antibody/antigen localization of the peptide, culetachykinin II (CTK II) was done with avidin–biotin staining kits (Pierce, Rockford, IL). The localization of the peptide was visualized with diaminobenzidine tetrahydrochloride (DAB) from Sigma (St. Louis, MO). The optimal dilution of CTK II was 1:2000 in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumen (BSA). Antibody specificity was confirmed by preincubation of the antiserum (1:1000) 2 h with synthetic CTK II at a concentration of 10 nmol/mL in the phosphate buffer. Histochemical results were visualized with an Olympus AH-2 compound microscope and photographed with Kodak Tmax-100 film. The results of the isolation, purification and structural characterization of CTK II has been reported in Meola et al., 1998.

Transmission Electron Microscopy

Heads of mosquitoes, containing the antennal segments were fixed in a solution of 2% glutaraldehyde, 2% paraformaldehyde, 2% acrolein, 1.5% dimethyl sulfoxide (DMSO), in 0.1 *M* sodium cacodylate buffer at pH 7.4 (Kalt and Tandler, 1971). Fixation occurred at room temperature for 6 h followed by refrigeration at 4°C for 16 h. Specimens were rinsed in 0.1 *M* cacodylate buffer three times, postfixed in 1% osmium tetroxide in 0.1 *M* sodium cacodylate buffer at pH 7.4 on ice at 4°C for 16 h. Osmium was removed with four washes of distilled water for 20 min followed by dehydration in a gradated ethanol series on ice. Following three rinses of 100% ethanol for 10 min each, the tissue was transferred through three changes (5 min each) of propylene oxide at room temperature and embedded in a mixture of araldite 502 and epon 812 (Mollenhauer, 1964). Ultrathin (50–70 nm) sections were poststained with 1% uranyl acetate in alcohol followed by Reynold's lead citrate (Reynolds, 1963). Sections were analyzed and photographed on a Zeiss 10 C transmission electron microscope at 80 kV using Kodak electron microscope film 4489 (Estar thick base).

Scanning Electron Microscopy

Abdomens, legs, and wings were removed and the thorax containing the heads and antennae were fixed 4 h in a solution of 3% glutaraldehyde and 2% paraformaldehyde and post-fixed in 1% osmium tetroxide for 1 h. Both fixatives were prepared in 0.1 *M* PBS at pH 7.2 (Pierce, Rockford, IL). After fixation, the tissues were rinsed several times in distilled water and dehydrated in a graded ethanol series, critical point dried, and then mounted on aluminum stubs with silver paint (Structure Probe Inc, West Chester, PA). The mounted specimens were coated with gold-palladium and viewed with a Cambridge 200 Stereoscan at 15 kV.

Terminology used for the sensilla is that of McIver (1982), whereas the terminology of the ultrastructure of the antennae was taken from Zacharuk (1985).

Results

Each antenna of a mosquito contains 13 flagellar segments, innervated by a pair of antennal nerves (Fig. 1A) in which the sensory nerves of the antennae extend to the antennal lobe of the deutocerebrum. Immunocytochemical localization of the peptide, CTK II revealed tachykinin-related material in five to six pairs of neurosecretory cells in the first flagellar segment (Fig. 1A) of both female and male mosquitoes. The axons of these antennal neurosecretory cells (ANC) could be traced within the antennal nerve (AN) to their terminals on glomeruli (G) of the antennal lobe where the ANC form neurotransmitter terminals on processes of the internuncial neurons comprising the neuropile of the glomeruli (Fig. 1B,C).

The first flagellar segment (FS) of the mosquito extends from the pedicel (P), which contains the Johnston's organ (JO), the main auditory organ of mosquitoes (Figs. 1A and 2A). Scanning electron microscopy of the antennae of *C. salinarius* revealed that the first flagellar segment is sexually dimorphic. In the male, the width of this segment is approx 35 µm at its base and 55 µm at its distal boundary, whereas the length is 85 µm. The portion of this segment containing the ANC appears distended (Figs. 1A and 2A). This enlarged region of the segment is covered with microtrichia (M) as is the remainder of the segment (Fig. 2A). In addition to the microtrichia, the lateral surface of this region contains a row of sensillae trichodia (large triangle) arranged in a semicircle, whereas a ring of larger sensillae chaetica (straight arrow), set in

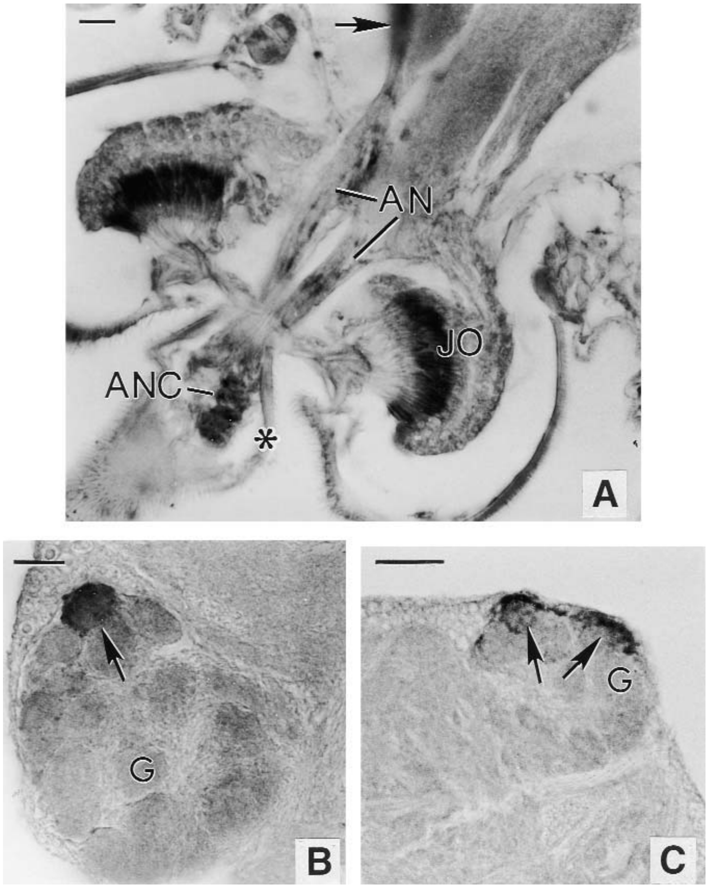

Fig. 1. Immunocytochemical localization of a culetachykinin (CTK II) revealed the presence of neurosecretory cells (ANC) in the first flagellar segment of both male and female *C. salinarius.* **(A)** The ANC extend axons along the paired antennal nerves (AN) to the antennal lobe (arrow). Asterisk indicates enlarged region of this segment in which the ANC are clustered. JO, Johnston's organ in pedicel. The CTK II-reactive axons of the ANC terminate (arrows) on specific glomeruli (G) of the antennal lobe in adult male **(B)** and female **(C)** *C. salinarius.* Bars = 10 µm.

sturdy sockets encircles the distal boundary of this segment (Fig. 2A, 2B). The first flagellar segment of the female is longer and uniform in width, measuring 130 µm in length and 50 µm in width (Fig. 2B). This segment in the female is densely covered with microtrichia (M), with sensillae trichodea scattered over its surface (Fig. 2B). The ring of large sensillae chaetica (straight arrow) found on the distal boundary of the male segment (Fig. 2A) is absent in the female; however, a ring of sensillae chaetica arises from the proximal boundary of the second flagellar segment (triangle) in the female (Fig. 2B). Both sexes have a cluster (broad arrow) of 6 scales on the medial surface of the first flagellar segment (Fig. 2A,B).

Ultrastructural analysis revealed that these antennal neurosecretory cells (ANC) which produce tachykinin are found only in the first flagellar segment of both sexes and are approximately 7–9 µm long and 5 µm wide. The cytoplasm of these cells is well supplied with free ribosomes, rough endoplasmic reticulum, Golgi complex, and mitochondria (Figs. 3 and 4). The ANC produces spherical to slightly ovoid, electron-dense elementary

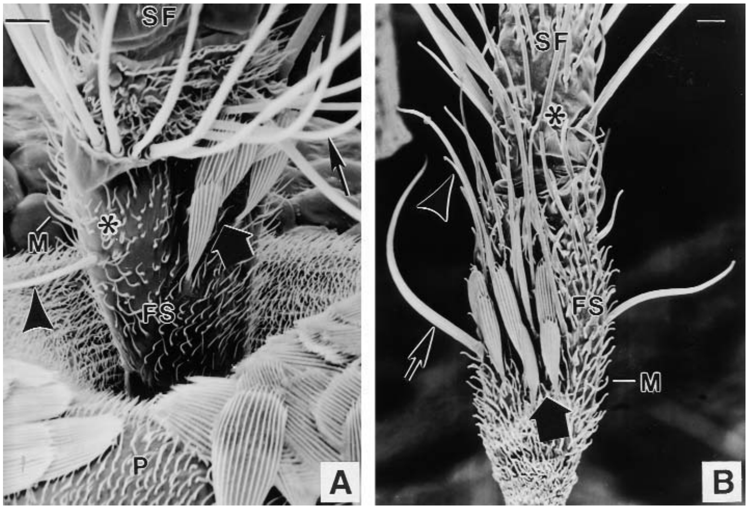

Fig. 2. **(A)** Scanning electron micrograph of the pedicel (P), first flagellar segment (FS), and base of second flagellar segment (SF) of an antenna of a male *C. salinarius.* This segment (FS) is urn-shaped, being narrow at its base and gradually increasing in width until it terminates in a ring of sensilla chaetica (narrow arrow) at its distal boundary. The expanded area (asterisk) contains the ANC, as seen in Fig. 1A. The surface of this segment is uniformly covered with microtrichia (M). Four of the six scales that project from the medial surface of this segment are visible (broad arrow) and one of the five sensillae trichodea of the dorsolateral surface is visible (arrowhead). **(B)** The width of the first flagellar segment of a female *C. salinarius* is uniform and relatively narrow throughout its length. Microtrichia cover this segment (M), and both sensilla chaetica (narrow arrow) and sensilla trichodea (arrowhead) are scattered over its surface. A cluster of scales are present on the medial surface of this segment (broad arrow). Large sensilla chaetica encircle the proximal boundary of the second flagella segment (asterisk). Bar = 10 µm.

neurosecretory granules (ENG), enclosed in a unit membrane (Figs. 3 and 4). These ENG are approx 140–220 nm in diameter. Unlike typical neurons, the ANC and their processes are not enclosed by a glial sheath (Figs. 3, 5, and 6), except when the perikaryon is in close contact with the antennal nerve (Fig. 3) or when the axon of one of the ANC enters an antennal nerve (Fig. 4). The ANC of the male and female differ in their relationship to the antennal nerve so that the ANC of the female is closely apposed to the antennal nerve (Fig. 3), whereas the ANC of the male are clustered adjacent to the antennal nerve (Fig. 4). The ANC of both sexes send axons into this nerve where they form numerous branches prior to terminating on specific glomeruli in the antennal lobe (Fig. 4). No synapses were found between neurosecretory axons and the sensory axons within the antennal nerve.

In addition to sending processes to the antennal lobe, the ANC of both sexes form collaterals (Fig. 5) that extend between the glial cells sheathing the sensory neurons (Figs. 6 and 7) and terminate on the plasma membrane of the receptor lymph channels (RLC) that enclose the sensory dendrites (Fig. 8). The RLC are formed by the sheath cells of the sensory neurons and are distinguishable from the sensory neurons by their electron-dense cytoplasm containing a large number of free ribosomes, electron-dense ENG, and relatively large number of mitochondria (Figs. 5–7). Small, electron lucent vesicles (arrow) along the inner plasma membrane of the portion of the collaterals terminating on the membrane of the RLC indicate release of the products of the ANC into the RLC (Fig. 8). The dual destination of the ANC terminals on the dendrites of the antennal sensilla and on the internuncials of the antennal glomeruli of the mosquito brain (Fig. 9B) is compared with a schematic of a typical insect antennal sensory system (Fig. 9A).

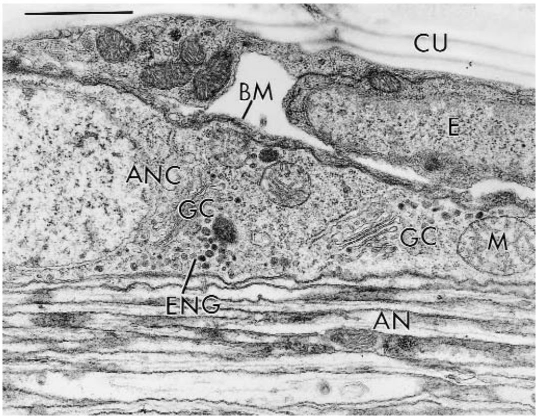

Fig. 3. Transmission electron micrograph of a longitudinal section through the first antennal segment of a female *C. salinarius,* revealing an antennal neurosecretory cell (ANC) closely apposed to the surface of an antennal nerve (AN). The ANC lie beneath the epidermal cells (E) that formed the antennal cuticle (CU). The free surface of the ANC is covered only by a basement membrane (BM). Small, electron-dense elementary neurosecretory granules (ENG) are visible in the cytoplasm of the ANC as well as several mitochondria (M) and golgi complex (GC). Bar = 1.0 µm.

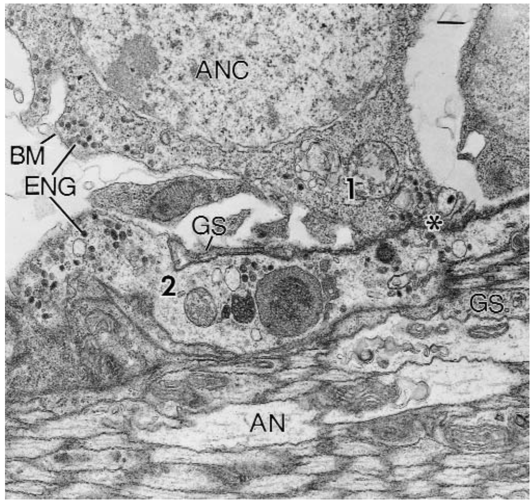

Fig. 4. Transmission electron micrograph of a longitudinal section through the first antennal segment of a male *C. salinarius.* The axon (1) of an ANC has extended to the surface (asterisk) of the antennal nerve (AN), while that of another ANC (2) has entered the antennal nerve. The electron-dense, elementary neurosecretory granules (ENG) of these cells are relatively small. Note, the absence of a glial sheath enclosing the perikaryon of the ANC, only a thin basement membrane (BM) covers these cells. GS, glial sheath of antennal nerve. Bar = 0.5 µm.

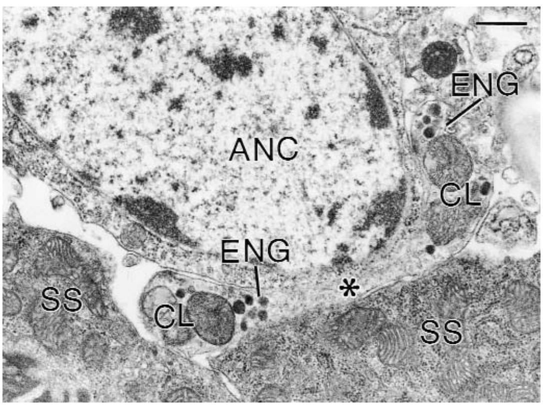

Fig. 5. Transmission electron micrograph of a tangential section through an antennal neurosecretory cell (ANC) at the region of paired collaterals (CL) containing ENG, extend from the axonal hillock (asterisk). Sheath cells (SS) that form the receptor lymph channel of the sensory dendrites are seen in close proximity to the ANC. Bar = 1.0 µm.

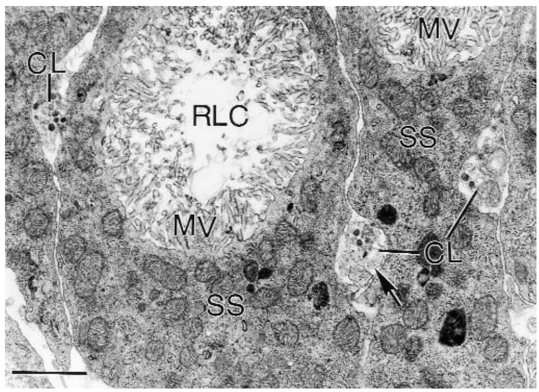

Fig. 6. Transmission electron micrograph of a row of sensory sheath cells (SS) of the sensory neurons of the antennae of *C. salinarius.* The microvilli (MV) of two of the sheath cells extend into the receptor lymph channels (RLC) formed by these cells. Three collateral processes (CL) of the antennal neurosecretory nerves can be seen extending between the sheath cells. Note that these processes, like the perikarya of the ANC, are devoid of glial sheaths (arrow). Bar = 1.0 µm.

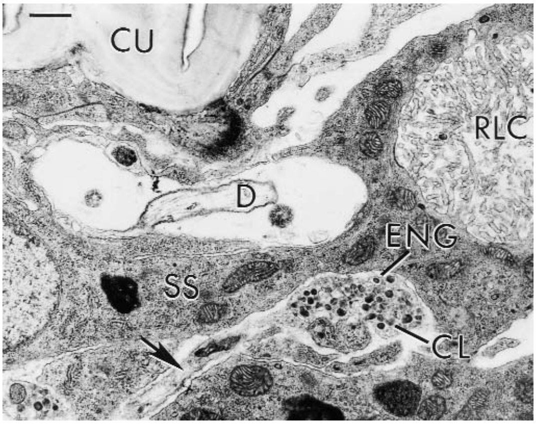

Fig. 7. Transmission electron micrograph revealing a collateral (CL) of an ANC in close proximity to a sensory dendrite (D) lying within the inner receptor lymph channel of a sensory sheath cell (SS) at the entrance of the dendrite into the cuticular portion of the sensillum. Both the sensory dendrite and the collateral of the ANC are surrounded by the glioplasm of the sheath cell, that extends microvilli into the outer receptor lymph channel (RLC). The terminal of the collateral is filled with electron-dense elementary neurosecretory granules (ENG), and a mitochondrion is visible in the process (arrow) of this collateral. CU, antennal cuticle. Bar = 0.5 μm.

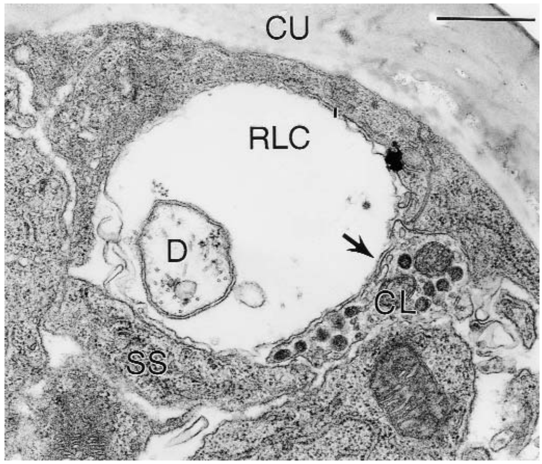

Fig. 8. Electron micrograph of transverse section through sensory cell dendrite (D) lying within receptor lymph channel (RLC) formed by the sensory sheath cells (SS). This portion of the sensilla lies just beneath the antennal cuticle (CU). The plasma membrane of a terminal of a collateral (CL) of an antennal neurosecretory cell has fused with the cell membrane forming the RLC and empty vesicles (arrow) lying adjacent to the fused membranes indicate release of neurosecretory material into the lymph surrounding the sensory dendrite. Bar = 0.5 μm.

Discussion

The antennae are the primary olfactory organs of mosquitoes and contain chemoreceptors that undergo changes in sensitivity that vary with the physiological state of the insect (Bowen, 1991). These changes in receptor sensitivity may well be affected by the tachykinin-related peptide at the level of the dendrites of the sensory cells or by the release of this peptide at their synapses on the glomerular interneurons of the antennal lobe. Tachykinins are widely distributed in vertebrates, where they have been found to be potent secretogogues, induce behavioral responses and vasodilatation, stimulate neurons and initiate smooth muscle contractions (Maggio, 1988). In insects, tachykinins have a myostimulatory activity on hindgut and oviduct muscle (Nassel, 1999), serve as interneurons that modulate the response of other neurons (Lundquist, 1994) and may be involved in development (Rosay et al., 1995).

Culetachykinin reactive neurons in the antennae of *C. salinarius* were found to contain typical membrane-bounded, electron-dense elementary neurosecretory granules at the lower range of size for neurosecretory granules (140–220 nm). The ANC have a relatively large nucleus containing scattered chromatin with some chromatin clumped along the inner nuclear membrane and relatively sparse karyoplasm surrounding the nucleus. The majority of the cytoplasm of these cells is utilized in the formation of neuronal processes. The main process or axon enters the paired antennal nerves that extend to the antennal lobe of the deutocerebrum. After entering the antennal nerves, the ANC form numerous branches that terminate on interneurons of specific glomeruli of the antennal lobe. In addition to these terminals in the brain, the ANC have collaterals that extend between the sheath cells of the antennal sensory neurons and form neurohemal release sites in the receptor lymph channels of the dendrites of the sensory neurons.

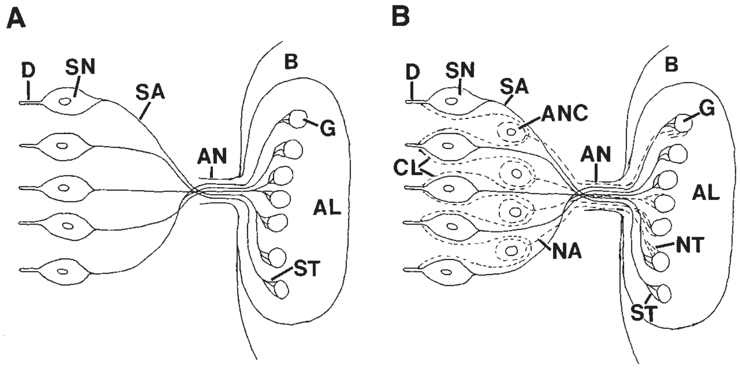

Fig. 9. Schematic diagram comparing a typical insect antennal sensory system with that of the mosquito, *C. salinarius* adapted from the vertebrate model by Axel (1995). **(A)** The typical antennal nervous system contains sensory neurons (SN) with dendrites (D) that extend through the cuticle, receiving sensory stimuli from the environment. This information is transferred along the sensory axons (SA) of the SN via the antennal nerve (AN) to the brain (B) where they terminate (ST) on interneuronal processes in glomeruli (G) of the antennal lobe (AL). **(B)** In *C. salinarius*, in addition to the typical sensory receptor system of other insects, the first flagellar segment also contains neurosecretory cells (ANC) that not only extend axons (NA) to the antennal lobe (AL) where they terminate (NT) on specific glomeruli (G), but the ANC also extend collateral processes (CL) to the region of the dendrites, where a tachykinin produced by the antennal neurosecretory cells (ANC) is released into the receptor lymph channels surrounding the sensory dendrites, thus modulating the behavior of these cells.

Thus, the ANC appear to have multiple functions that require both neurotransmitter and neurohemal modes of release. The release of multiple peptigenic messengers at different areas of the body from a single neurosecretory cell has been well-documented in insects (Schoonveld, 1991; Blackburn et al., 1992). As for the regulation of the synthesis and release of peptigenic products of the ANC, these cells are bathed in hemolymph that is continuously circulated with that of the main body of the insect via antennal pulsatile organs (Jones, 1977) and in this manner receive feedback from the functions they mediate. In addition, the ANC are located in peripheral organs that are enclosed by a relatively thin cuticle and thus may be directly influenced by photoperiod.

Thus far, the only other species of insect in which neurosecretory cells have been found to form synapses on the glomeruli of the antennal lobe is the blowfly, *Calliphora vomitoria* (Lundquist et al., 1994). These cells also produce a tachykinin-related peptide, but their perikarya are located in the deutocerebrum lobes of the brain and they function as interneurons, whereas the ANC of *C. salinarius* are peripheral neurosecretory cells that appear to have both a neurotransmitter and neurohemal modes of release. In any case, the presence of these cells in a peripheral organ in close proximity to sensory cells that have such a vital impact on the survival and reproduction of the insect indicates the importance of the mediatory effect of the ANC on these vital functions and the rapidity of the response required by their products on their target organs.

Ultrastructural analysis of the antennae of other genera of mosquitoes needs to be done to determine if ANC are characteristics of just this species of mosquito or of this family of Diptera. If neurosecretory cells can be found in other genera of mosquitoes, they would be targets for development of mimetic peptides that would serve as a species-specific means of controlling some of our most serious insect vectors.

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